Antineoplastic activity of taurolidine and its derivatives on human ex vivo glioblastoma bulk cells and cancer stem cells

Abstract

Despite multimodal therapy, patients suffering from glioblastoma (GBM) still have a dismal prognosis. The identification of cancer stem cells (CSC) in brain tumour tissue, yielded hope that the vulnerable target to combat GBM has been found. Several study groups worldwide concentrate nowadays on therapeutic strategies that effectively target CSC. Since in our laboratory has been revealed that taurolidine, a derivate of the amino acid taurin, displays a potent antineoplastic effect in human glioma cell lines and in ex vivo malignant cell culture, this thesis focused on the susceptibility of glioblastoma CSC to taurolidine and temozolomide (TMZ), the most commonly used chemotherapeutic agent so far. Additionally, we investigated the most powerful derivative of taurolidine and tested its antineoplastic activity on CSC as well.

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Antineoplastic Activity of Taurolidine and its Derivatives on Human ex vivo Glioblastoma Bulk Cells and Cancer Stem Cells

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Abbreviations

BTSC	brain tumour stem cells
CSC	cancer stem cells
CXCR1	IL-8 receptor
EGF	epidermal growth factor
FasL	Fas ligand
FCS	fetal calf serum
FGF	fibroblast growth factor
GBM	glioblastoma multiforme
HBSS	Hank’s bilance salt solution
LIF	leukemia inhibitory factor
MGMT	methyl-guanine methyl transferase gen
NAC	N-acetyl-L-cysteine
NCS	neural stem cell
ROI	reactive oxygen intermediates
SCID	severe combined immunodeficiency
TMZ	temozolomide
TT	taurultam
Z-VAD-FMK	pan-caspase inhibitor

1. Summary

Objective: Despite multimodal therapy, patients suffering from glioblastoma (GBM) still have a dismal prognosis. The identification of cancer stem cells (CSC) in brain tumour tissue, yielded hope that the vulnerable target to combat GBM has been found. Several study groups worldwide concentrate nowadays on therapeutic strategies that effectively target CSC. Since in our laboratory has been revealed that taurolidine, a derivate of the amino acid taurin, displays a potent antineoplastic effect in human glioma cell lines and in ex vivo malignant cell culture, this thesis focused on the susceptibility of glioblastoma CSC to taurolidine and temozolomide (TMZ), the most commonly used chemotherapeutic agent so far. Additionally, we investigated the most powerful derivative of taurolidine and tested its antineoplastic activity on CSC as well.

Methods: Studies were performed using different glioblastoma cell lines and isolated ex vivo glioblastoma cell cultures. CSC were isolated from tumour tissue by the use of neurosphere culture conditions. We investigated the antineoplastic potential of taurolidine, its eight derivates and TMZ on these cells by performing cytotoxicity assays.

Results: Our findings revealed that all tested glioblastoma CSC were sensitive to taurolidine and taurultam (TT). TT was the most powerful taurolidine derivate of the eight ones tested. The mean EC_{50} of taurolidine was 13 ± 2 μg/ml and the mean EC_{50} of TT was 11 ± 0.9 μg/ml, both in the range of the measured plasma level of patients treated with taurolidine. In contrast, the mean EC_{50} of TMZ was 69 ± 26 μg/ml, 5-fold higher than its peak plasma level.

Conclusion: Targeting and eliminating CSC is a prerequisite to improve outcomes for GBM patients. According to our findings that CSC are sensitive to taurolidine and its most powerful derivative TT, both of them are promising new therapeutic agent in the GBM therapy and should be investigated in a clinical trial.

2. Introduction

The most frequent primary brain tumours in adults are gliomas [1]. Amongst them, glioblastoma multiforme (GBM) are the most common and, due to its invasiveness and propensity for recurrence, the most malignant tumours. Despite optimal treatment and intensive ongoing research, the outcome remains poor. The median survival is only 12 to 15 months [2] and the 2-year survival rate is 10-25% [3]. The identification of cancer stem cells (CSC) in human brain tumours by Singh and his colleagues [4], yielded hope that the vulnerable target to combat GBM has been found. CSC are defined as a small subpopulation of tumour cells, which are able to self-renew, proliferate and generate tumours [3]. Several study groups worldwide focus now on therapeutic strategies that effectively target CSC. It is essential to overcome their resistance to radio- and chemotherapeutics if glioblastomas shall be completely eradicated [2].

2.1 Glioblastoma multiforme

2.1.1 Epidemiology

The annual incidence of malignant gliomas is approximately 5 cases per 100.000 people. GBM account for approximately 60 to 70% of malignant gliomas, anaplastic astrocytomas for 10 to 15%, and anaplastic oligodendrogliomas for 10%, less common tumours account for the rest. The incidence has increased slightly over the past two decades, primarily as a result of improved diagnostic imaging. The median age of patients at the time of diagnosis is 64 years and men are more frequently affected than women (sex ratio 3:2) [2, 5].

2.1.2 Etiology

No underlying cause has been identified for the majority of malignant gliomas. The only established risk factor is exposure to high doses of ionizing radiation. An association with head injury, occupational risk factors or exposure to electromagnetic fields couldn't be proved. 5% of patients with malignant gliomas have a family history of gliomas. Some of these familial cases are associated with rare genetic syndromes, such as neurofibromatosis types 1 and 2, the Li-Fraumeni syndrome and Turcot's syndrome. Nonetheless, most familial cases have not an identified genetic cause [2].

Glioblastoma multiforme either develop de novo (primary GBM>90%) or by progression from low-grade or anaplastic astrocytomas (secondary GBM = 5-10%) [5].

2.1.3 Localization and tumour growth

The most common localization is the deep white matter of the cerebral hemispheres, particularly in the frontotemporal regions. Due to its infiltrative growth, GBM rapidly extends into the cortex or the basal ganglia causing clinical symptoms. As it can across the corpus callosum into the contralateral hemisphere, it is also termed butterfly glioma. GBM of the brainstem, cerebellum or spinal cord hardly occur [5].
Although extremely malignant, extra-cranial metastasis from GBM are quite rare with an incidence of <2% reported in the published literature. In general, they are asymptomatic or are found only at autopsy. Factors contributing to the limited incidence of metastatic disease include the dense impassable dura, the unique extracellular matrix of the brain, the tough basement membrane that surrounds intracerebral blood vessels, the lack of true lymphatic vessels and limited patient survival. The regional extension and less frequently lepto-meningeal dissemination are the main causes of death [1].

2.1.4 Clinical findings

Four main circumstances lead to arise the presumptive diagnosis. First, patients have partial or generalised seizures. Second, raised intracranial pressure caused by vasogenic edema provokes headache, nausea, vomiting, drowsiness and visual abnormalities (diplopia due to abducens-nerve palsy). Classic headaches that are suggestive of increased intracranial pressure are most severe in the morning and may awake the patient from sleep [2]. Third, progressive focal neurological deficits generally show the tumour side. Supratentorial tumours induce motor- or sensitive deficits while posterior fossa tumours are associated with various combinations of cranial-nerve palsies, cerebellar dysfunction and long-tract signs. Finally, cognitive dysfunction of variable severity represents the frontal or diffuse brain infiltration [1].
Patients with a de novo GBM normally present with a short clinical history whereas secondary GBM patients have in general a clinical history over years.

2.1.5 Histology

Analysis of the most malignant region of the tumours permits grading dependent on main features such as high cellularity, pleomorphism, nuclear atypia, mitoses, microvascular proliferation and necrosis [1]. On the basis of these histological features, the World Health Organization classifies astrocytomas into four prognostic grades: pilocytic astrocytomas (grade I), diffuse astrocytomas (grade II), anaplastic astocytomas (grade III) and glioblastoma multiforme (grade IV), which is the most frequent subtype [2].
Macroscopically, GBM appears poorly delineated, with greyish vital and yellowish necrotic areas. Multiple areas of old and recent haemorrhages are also typical [5].

2.1.6 Radiographic findings

Magnetic resonance imaging (MRI), with and without gadolinium infusion, is the imaging technique of choice when a brain tumour is clinically suspected [1]. There, GBM typically presents as heterogeneously enhancing mass with surrounding edema. Glioblastomas frequently have central areas of necrosis and extensive peritumoral edema [2]. Mass effect in form of a midline shift, caused by the rapidly growing tumour, is very often seen [5]. If no MRI is available, CT scanning, although less sensitive than MRI, is appropriate to obtain a quick assessment of the lesion [1]. Further imaging techniques as functional MRI or diffusion-weighted imaging are increasingly used as diagnostic aids and to monitor the response to therapy [2].
However, imaging patterns are not specific and diagnosis must be confirmed by histological examination of tumour biopsy samples or by surgical resection. As morphological assessment might be confusing, it is important that clinical, radiological and pathological data are in accordance [1].

2.1.7 Therapy and prognosis

General management
Glioblastomas are still incurable and the aim of the palliative treatment is to improve neurological deficits and to increase survival while maintaining the best possible quality of life. Much of the care of patients with GBM involves general medical management and psychological support. The most common problems include seizures, peritumoural edema, venous thromboembolism, fatigue, cognitive dysfunction and depressions. Patients presenting seizures should be treated with antiepileptic drugs. Since they can have negative

interference with chemotherapy, a strict indication is essential. To treat peritumoural edema, corticosteroids are frequently used. Because of their multiple side-effects and their influence on chemotherapy, only the minimum efficient dose should be prescribed. While the patients have an increased incidence of venous thromboembolism from leg and pelvic veins, they sould get anticoagulation therapy assuming there is no contraindication such as intracerebral hemorrhage. Depression is underdiagnosed in patients with malignant gliomas and antidepressants and psychiatric support are often invaluable. Rehabilitation, including physical therapy, should not be discouraged by a presumed poor outlook [1] [2].

Specific management

The standard therapy for newly diagnosed GBM involves maximal surgical resection, radiotherapy and chemotherapy. Surgical debulking reduces the symptoms from mass effect and provides tissues for histological diagnosis. Due to their infiltrative nature, GBM cannot be completely eliminated surgically. Therefore, adjuvant radiotherapy and chemotherapy are essential. The addition of radiotherapy to surgery increases survival from a range of 3 to 4 months to a range of 7 to 12 months. Chemotherapy is assuming an increasingly important role in the treatment of malignant gliomas. Recent research results demonstrated that the most successful adjuvant therapy for newly diagnosed GBM consists of a radio - and chemotherapy with temozolomide (TMZ): TMZ is given concomitantly with radiotherapy, followed by six monthly cycles of TMZ. Despite continued advances in surgical and medical therapies, prognosis with a 5 year mortality rate of close to 100% remains dismal and further improvements are desperately needed [2] [5].

2.2 Cancer stem cells

2.2.1 History

A study of leukaemia was the first to report that a specific subpopulation of leukemic cells was capable of initiating human AML after transplantation into SCID mice. These SCID leukaemia-initiating cells (CD34+/CD38-) posses differentiative and proliferative capacities and the potential of self-renewal that are not present in the majority of leukemic cells [6, 7]. Furthermore, it was shown that this subpopulation is necessary and sufficient to generate or maintain the tumour population [6].

Meanwhile similar CSC have been described for a wide variety of solid tumours including brain tumours, breast cancer, bone sarcoma, colon cancer, liver cancer, lung cancer, and melanoma [3, 6].

2.2.2 Brain tumour stem cells (BTSC)

In 2003, the identification and purification of CSC from human brain tumours that posses a marked capacity for proliferation, self-renewal and differentiation was described by Singh and colleagues [4]. BTSC was isolated by selecting cells expressing the neural stem cell surface marker CD133. These CD133+ cells could differentiate in vitro into tumour cells that phenotypically resembled the tumour from the patient. The self-renewal capacity of tumour cells was only present in the CD133+ fraction. The authors suggested there is coherence between the self-renewal capacity of the brain tumour stem cells and the increased malignancy. Injection of as few as 100 CD133+ cells into mice produced a tumour, whereas injection of 10^5 CD133- could not induce any tumour progression [3].
Concomitantly Galli and his colleagues reported an alternative strategy based on the neurosphere assay to isolate BTSC [8]. They found that cells dissociated from human brain tumour tissue proliferate and expand exponentially under the culture conditions used to grow normal neurospheres [3]. It has further been proved by Liu and others that the percentage of CD133+ was significantly elevated in recurrent gliomas than that in autologous primary tumour tissue [9].
A BTSC is defined as follows: a cell must be able to 1) generate clonally derived cells that form neurospheres 2) self-renew and proliferate 3) differentiate and express typical markes of brain cells 4) induce tumours after in vivo transplantation in animal models that are similar to the donor patient's original tumour [6].

2.2.3 Origin of brain tumour stem cells

The cell origin of brain tumours remains a subject of ongoing debate in the current scientific literature [10]. Two main hypotheses coexist: 1) brain tumours arise from the transformation of a normal stem cell (NSC) or 2) brain tumours arise from the dedifferentiation of a mature brain cell due to genetic alterations (see Fig. 1) [6]. There is, however, evidence to support a combined role for NSC transformation and dedifferentiation of neuroepithelial precursor cells [3]. On one side several lines of evidence suggest that brain tumours arise from the transformation of a normal NSC, all of which rely on the recognition of the many functional and genetic similarities shared by somatic stem cells and cancer cells. Furthermore, brain tumours can be very heterogeneous, being comprised of cells expressing phenotypes of more than one neural lineage, implicating a multipotential cell of origin. On the other side, there exist data supporting that dysregulation of specific genetic pathways, rather than cell of origin, may dictate the emergence and phenotype of high-grade gliomas. In summary, a NSC may be seen as a more likely compartment for transformation, since its self-renewal

machinery is already primed and it has a long lifespan favouring the accumulation of mutations [10].

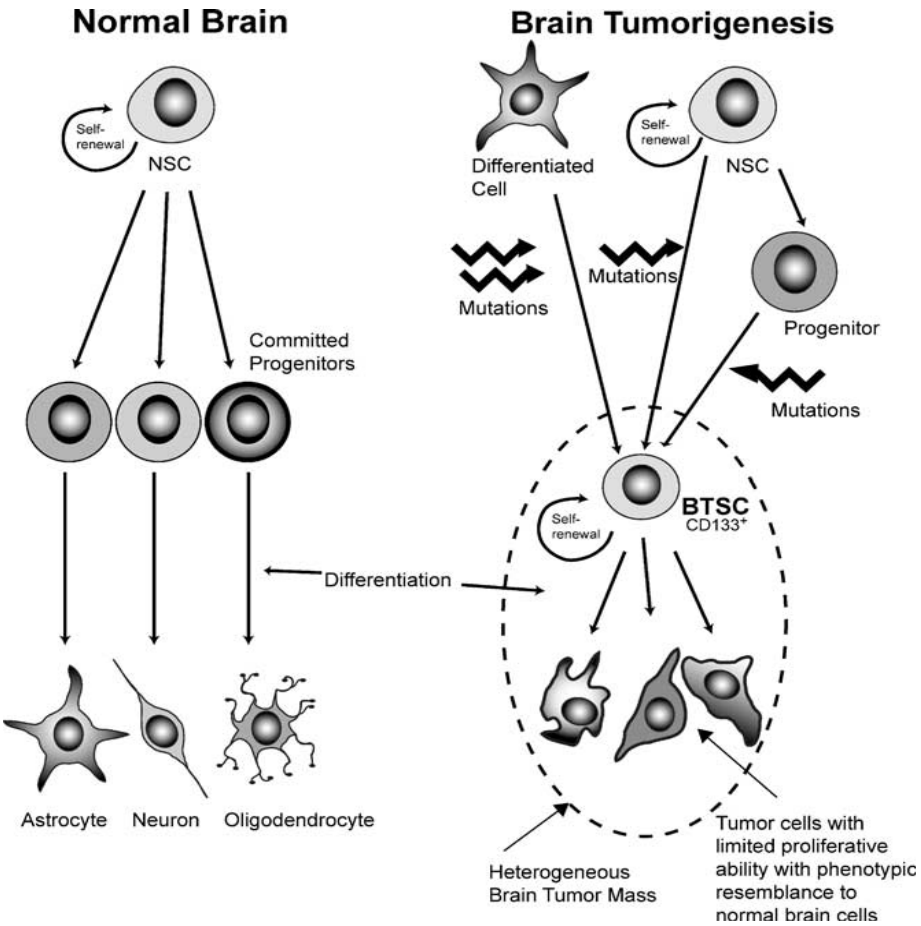

Figure 1. On the left, normal neural stem cells in the brain undergo highly regulated self-renewing divisions to regenerate themselves. In addition, they also generate lineage-committed progenitor cells and then differentiate into the three main neural lineages, neurons, astrocytes and oligodendrocytes. On the right, multiple mutation events target a stem cell, progenitor cell or mature brain cell resulting in the formation of a BTSC (see Ref. [10])

2.2.4 Therapeutic impact

The fact that a small population of highly potent cells allows the tumour to grow, might explain the recurrence of some brain tumours after surgery, chemotherapy and radiation therapy. These treatment failures are nowadays seen as the persistence of CSC [3]. Different mechanism underlying CSC chemo- and radiorestistance have been postulated [3] [11] [12]. Radioresistance in stem cells mainly results from the preferential activation of DNA-damage-response pathways, whereas chemoresistance results from the overexpression of MGMT, the up-regulation of multidrug resistance genes, and the inhibition of apoptosis [2]. Therefore new therapeutic research should focus on the eradication of CSC, increasing their sensitivity to chemotherapy and radiation therapy.

2.3 Temozolomide

2.3.1 Features of temozolomide

Temozolomide (TMZ) is the most commonly used chemotherapeutic agent in the therapy of GBM. In 2005, Stupp and colleagues proved that the addition of TMZ to radiotherapy after debulking surgery resulted in a clinically meaningful and statistically significant survival benefit [13]. They reported that the combination of radiotherapy and TMZ compared with radiotherapy alone increases the median survival (14.6 months vs 12.1 months) and the 2-year survival rate (27% vs 10%). Recently, these authors published long-term results from their research proofing the survival advantage of combined treatment but also confessing that long time survivors are still rare [14].

TMZ achieves its cytotoxic effect mainly by methylating the O^6 position of guanine, which mismatch with thymine leading to cancer cell death [9] [15]. The DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT) can remove O^6-methylguanine. MGMT is only expressed in a subgroup of GBM. Therefore, TMZ receives its highest efficacy against tumours lacking MGMT expression due to a methylated MGMT promoter [16]. It is of note that on genetic modification of the MGMT in the course of tumour development results in increased susceptibility in about 20% of the patients [17]. So the MGMT promoter methylation is not only a prognostic marker, but also a predictive marker for response to TMZ. This molecular marker could become in future an indicator for treatment decision [18].

2.3.2 The effect of temozolomide on CSC

There are controversial findings of TMZ on CSC. Beier and others found that TMZ preferentially eliminates CSC but spares more differentiated tumour cells [16] whereas Liu and colleagues reported that CD133+ cells had significant resistance to TMZ compared to CD133- cells due to higher MGMT expression in CD133+ cells [9].

2.4 Taurolidine

2.4.1 Features of taurolidine

Taurolidine is originally developed in the 1970s by Geistlich-Pharma AG (Wolhusen, Switzerland). It is chemically identified as Bis-(1,1-dioxoperhydro-1,2,4-thiadiazinyl-4) methane and represents a derivative of the amino acid taurin [5].

Taurolidine has been shown to exhibit antimicrobial and antiadherence properties [19]. Clinically, taurolidine is established as an antimicrobial agent and mainly used as an antibacterial lavage in abdominal surgery to reduce postoperative infection or postoperative peritoneal adhesion. It is also used to treat local or diffuse peritonitis [5]. Its mechanism as an antibiotic agent seems to be related to a chemical reaction between the active taurolidine breakdown products and structures on the wall of bacteria, resulting in disruption of bacterial cell wall and bacterial cell adhesion. In addition, it neutralizes endotoxins and lipopolysaccarides released by bacteria. The ability of taurolidine to affect cell surface structures and function explains its antiadherence properties [20].

Recently, at doses considerably lower than required for bactericidal activity, taurolidine was found to posses also antineoplastic and antiangiogenic activities [19]. The significant and selective antineoplastic activity has been proved for several human tumour cell lines as GBM, colon, lung, ovarian, prostate and melanoma [5] [20]. The antineoplastic effect of taurolidine is not only attributed to classic apoptosis, but also to other death pathway mechanism. Our previous studies revealed that taurolidine-induced cell death was induced by the formation of reactive oxygen intermediates (ROI) which were abolished by the radical scavenger N-acetyl-L-cysteine, but not by the pan-caspase inhibitor Z-VAD-FMK [19]. Stendel, et al could demonstrate that through induction of oxidative stress more than one cell death mechanism is involved, naming different caspase-independent programmed cell death pathways such as autophagy, senescene, necroptosis and necrosis [21]. The antiangiogenic ability is due to the potential of taurolidine to decrease the amount of secreted vascular endothelial growth factor (VEGF) by the tumour cells. As VEGF is said to be a critical regulator of tumour expansion and neovascularization, taurolidine could suppress tumour cell growth by its combined antineoplastic and antiangiogenic effects [19].

2.4.2 Features of the derivatives of taurolidine

As shown in Figure 2, taurolidine is broken down into taurinamide and finally, into taurin and water with reactive methylol groups being formed as intermediates [19]. The derivatives have been produced, purified and provided by Geistlich-Pharma AG.

They are coded in the following manner:

- TT = Taurultam
- 2012 = Dimethyltaurultam
- GS208 = N-Methyltaurultam
- HG 40
- E1234
- 2240
- 1183A
- 1183BX

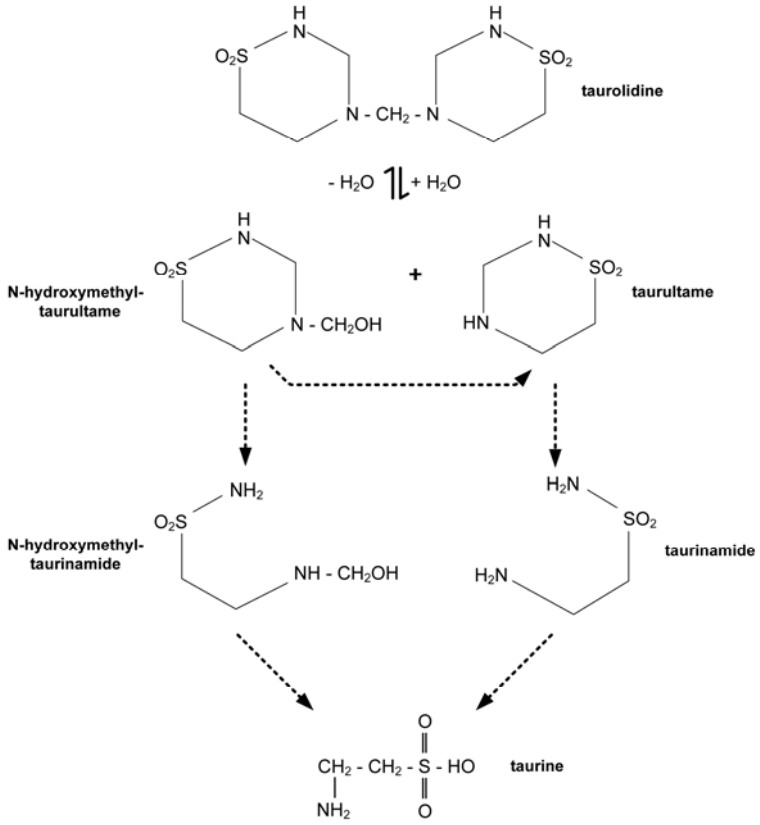

Figure 2. Simplified scheme of the formation of some derivates of taurolidine. [22]

Considering the high antineoplastic activity of taurolidine on human glioma cell lines, we decided to investigate all the intermediates on their antineoplastic properties. Thus, the main goal of this study was to find the most powerful derivate of taurolidine regarding the fact that isolating the responsible intermediates could reduce the clinical side effects of taurolidine. As the chemo- and radioresistance of CSC is seen to be responsible for the treatment failures, our further interest focused on the effect of taurolidine and its derivatives on CSC.

We first performed cytotoxicity assays after 24 and 48 hours of exposure to taurolidine and its derivatives on several human glioma cell lines and ex vivo glioblastomas. From our previous studies we knew that taurolidine induces cell death through the generation of reactive oxygen intermediates (ROI). We elucidated that the most powerful derivate TT

performed cell death by ROI induction. Because we were interested in the effect of taurolidine and TT on CSC, we tested in addition their cytotoxicities on mouse and human CSC. We also included TMZ, the most commonly used chemotherapeutic agent in the GBM therapy, in our investigations. Based on earlier findings that GBM and glioma cell lines express IL-8 and their corresponding receptors [23] and a recent report [24] showing that on anti-CXCR1 antibody induces cell death, we tested the blockage of IL-8 receptor (CXCR1) by an anti-CXCR1 antibody on CSC survival and the CSC initating capacity by the addition of recombinant IL-8.

3. Material and Methods

3.1 Reagents

Taurolidine stock solution of 20 mg/ml and its derivatives (2012, GS208, TT, HG40, E1324, 2240, 1183A, 1183BX) ultrapure powder were kindly provided by Dr. R. Pfirrmann (Geistlich Pharma AG, Wolhusen, Switzerland). For each experiment dilution series were freshly prepared in complete tissue culture medium at final concentrations ranging from 3.125 to 800 μg/ml. Before their use they were sterile filtered (0.22μm Acrodisc filter).
N-acetyl-L-cysteine (NAC), TMZ and crystal violet were purchased from Sigma Aldrich (Buchs, Switzerland). Z-VAD-FMK was bought from BioVision (Mountain View, CA, USA). Mega FasL was purchased by Apoxis SA (Lausanne, Switzerland). Recombinant IL-8 was obtained by PeproTech (Hamburg, Germany) and anti-CXCR1 antibody was purchased by R&D Systems (Minneapolis, USA).

3.2 Cell lines

The human glioma cell lines LN229, U373, LN18, U251 and U87G were kindly provided by Prof. N. de Tribolet (Geneva, Switzerland). T98G was obtained by American Type Culture Collection (Rockville, MD, USA). SMA 560 cells were kindly provided by Prof. D. D. Bigner (Durham, NC). The cells were cultured as monolayers in 75 cm^2 flasks (TPP, Trasadingen, Switzerland) in Dubecco modified Eagle's medium (DMEM, GIBCO, Invitrogen AG, Basel, Switzerland) containing 5% fetal calf serum (GIBCO), 2 mM N-acetyl-L-alanyl-L-glutamine (Biochrom, Berlin, Germany) and 20 μg/ml gentamycin (GIBCO) and maintained at 37°C in a 92% air/8% CO_2 humidified atmosphere. Cells were detached and splitted by using Trypsin-EDTA (GIBCO).

3.3 Patients

Human high grade gliomas were acquired from 6 patients undergoing surgery for brain tumour resection at the University Hospital Zurich, Switzerland. All tumour samples were high grade gliomas WHO Grade IV, all of them newly diagnosed. The diagnoses were confirmed at the Institute of Neuropathology, University Hospital Zurich. The median age of the patients was 54 ± 10 years (range 36-66 years) with a 4:2 male/female distribution. For all assays with ex vivo gliomas early passages of cultures (1-15) were used.

3.4 Dissociation of brain tumours

After tumour removal, tissues were immediately placed in petri dishes, minced mechanically in HBSS (GIBCO), and digested enzymatically by adding collagenase/dispase (Roche Applied Science, Rotkreuz, Switzerland) and DNase. For 1 hour this mixture was left on a magnetic stirrer in a 37° water bath. Thereafter, the dissociated cells are sequentially filtered through 70- and 100 μm cell strainers (BD Falcon, BD Biosciences, Basel, Switzerland) to remove any tissue debris. Erythrocytes were removed by resuspending and incubation the cells in ACK buffer (17 mM Tris-HCl [ph 7.2] containing 144 mM NH_4Cl) for 10 minutes at room temperature. The cells are washed in HBSS and after counting placed in culture.
The isolated tumour cells were either cultured as bulk in the same medium as described above or in neurosphere conditions using neurobasalmedium (GIBCO) containing 2% B-27 (GIBCO), 2 mM N-acetyl-L-alanyl-L-glutamine (Biochrom), 50 μg/ml gentamycin (GIBCO), 3.2 U/ml Heparin (Sigma Aldrich), 0.02 μg/ml EGF (PeproTech), 0.02 μg/ml FGF (PeproTech) and 2 ng/ml LIF (Sigma Aldrich). Only CSC survived in flasks containing neurobasalmedium. The bulk cells were cultivated in the same medium (DMEM) as used for human glioma cell lines.

3.5 Cytotoxicity assay

Tumour cells were cultivated as monolayers in 75 cm^2 flasks containing cell culture medium (see above). They were incubated at 37° until they were confluent for more than 80%. Then cells were washed with HBSS and separated by adding Trypsin-EDTA for 5 min. After centrifugation, dissolution with medium and cell counting, a suspension of 20 x 10^5 cells/ml was prepared. 20.000 cells per well were distributed in 100 μl to 96-well F-plates (BD Falcon). After overnight culture, the cells reached a confluence of 80% and were treated with taurolidine and its derivates. Therefore, taurolidine and each of its derivatives 2012, GS208, TT, HG40, E1234, 2240, 1183A, 1183BX were freshly prepared for each experiment at a concentration of 800 μg/ml. The solution was sterile filtred and further titrated to final concentrations of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 μg/ml. The final assay volume per well was 200 μl and for each sample tested triplicates were performed. The cells were incubated for 24 h, and after optical judgement, the remaining adherent viable cells were stained by adding 100 μl crystal violet solution (0.5% crystal violet in 20% methanol and 80% distilled water) for 15 minutes, washed in tap water, and air dried. Cell viability was quantified by measuring the absorbences at 540 nm using Titertek Multiscan plate-reader (Flow, Rockville, MD, USA). Cell survival is expressed as the percentage of the cells surviving relative to the number of cells of untreated control cultures. The killing rate was calculated

using the following formula: 100%-% survival. EC_{50} implies the concentration of drug that resulted in 50% killing.

3.6 Neurosphere culture

In parallel to bulk cultures, CSC were isolated from glioblastoma tissue using neurosphere culture conditions as described from Galli and his colleagues [8]. CSC were cultivated in 75 cm^2 flask containing complete neurobasalmedium. They were incubated at 37° until they formed neurospheres measuring approximately 150 μm. Neurospheres were splitted by using Trypsin-EDTA for 5 minutes, followed by the addition of 100 μl trypsin inhibitor (10 mg/ml, Sigma Aldrich). Thereafter, the cell suspension was filtered through a 30 μm cell filter (Partec, Münster, Germany). After centrifugation (700rpm for 5 minutes), the cells were resuspended with medium and counted to prepare a suspension of 10^5 cells/ml. Afterwards, cells were distributed in 100μl to 96-well F-plates (10.000 cells/well). 24 hours later, the CSC were treated with taurolidine, TT, TMZ, anti-CXCR1 antibody or recombinant IL-8. For each experiment, these agents were freshly prepared and diluted in complete neurobasalmedium. Taurolidine and TT were prepared at the concentrations of 1.56, 3.125, 6.25, 12.5 and 25 μg/ml. TMZ was concentrated between 5, 25, 250, 500 and 1000 μM. The final concentration of anti-CXCR1 antibody was 2.5, 10 and 25 μg/ml and recombinant IL-8 was 2.5, 10 and 25 ng/ml. The final assay volumes per well were 200 μl and each sample was tested at least three times. The cells were incubated for 7 days, and after optical judgement, 25 μl AlamarBlue (Invitrogen, California, US) was added per well. After 3-4 hours, the fluorescence, as a dimension of viability, was measured on a fluorescence spectrophotometer (Cytofluor 2350, Witec AG, Littau, Switzerland) at an excitation of 590 nm and an emission of 530 nm. Cell survival, killing rate and EC_{50} were calculated using the same formula as described above.

To compare the cytotoxicity of the mentioned agent on bulk cells, we treated the bulk cells in analogous way.

4. Results

4.1 Dose- and time-dependent cytotoxicity of taurolidine and its derivates on different cell lines and ex vivo cultured glioblastoma cells

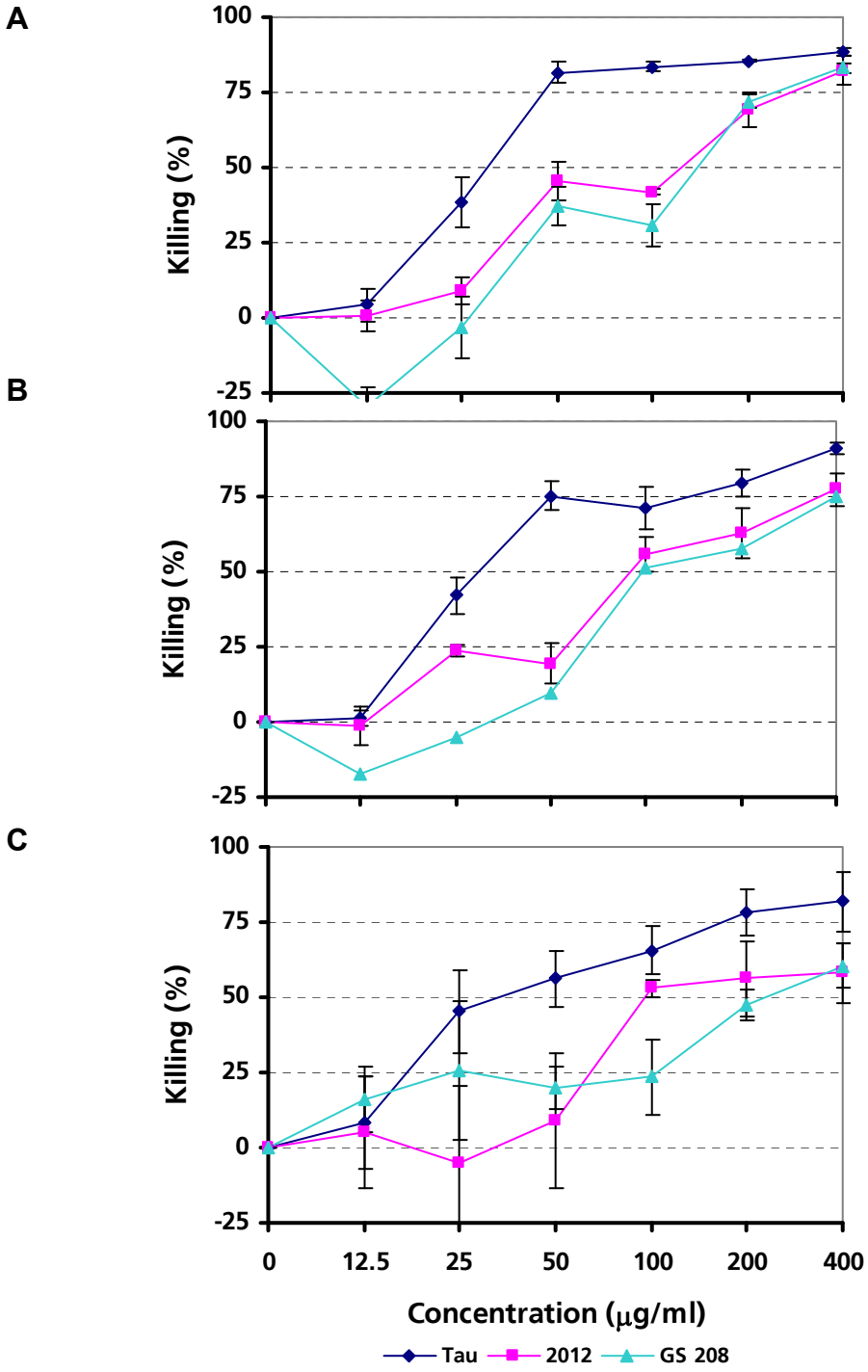

Figure 3. Taurolidine, 2012 and GS208 induced cytotoxicity in LN229 (A), U373 (B), U251 (C) glioma cells. 20.000 cells/well were treated for 24 hours and cytotoxicity was evaluated by crystal violet staining as described in Material and Methods. Data are presented as mean values ± SD of three independent experiments.

Six glioblastoma cell lines and two ex vivo glioblastoma cell cultures were tested for their sensitivity to taurolidine and its derivates (2012, GS208, TT, HG40, E1234, 2240, 1183A, 1183BX) in the range of 3.125 μg/ml to 800 μg/ml. Figure 3 demonstrates the percentages of cells that were killed (killing%) after 24 hours of exposure to taurolidine and its derivates 2012 and GS208 in cell lines LN229, U373, U251. As shown in Figure 3, cytotoxicity of these agents displayed a dose-dependency.

In these cytotoxicity assays the effective concentration of taurolidine required to kill 50% of the cells (EC_{50}) was remarkable lower than EC_{50} of 2012 and GS208 (Fig. 4A). An increase of the exposure-time to 48 hours did not reduce EC_{50} significantly (Fig. 4B).

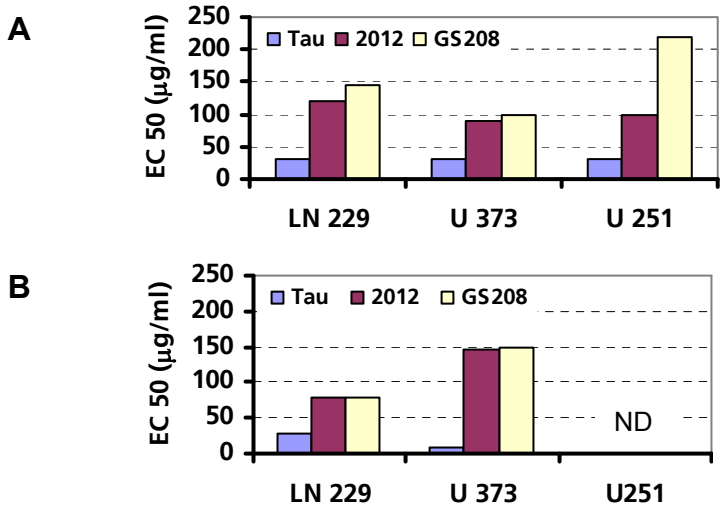

Figure 4. EC_{50} of taurolidine, 2012, GS208 in LN229, U373 and U251. Cytotoxicity was assessed after 24 h (A) and 48 h (B) of treatment. Data are shown as mean values of three individual experiments. ND=not done.

Remaining derivatives which were tested (TT, 1183A, 1183BX) are presented in Figure 5. EC_{50} of TT is comparable with the value of taurolidine. To answer the question of whether freshly isolated human glioblastoma cells are also sensitive to taurolidine or to any of its derivates, we established cell cultures of two freshly resected tumour tissues and performed cytotoxicity assays. Also freshly isolated human glioblastoma cells showed susceptibility to taurolidine and TT in a similar range (Fig. 5).

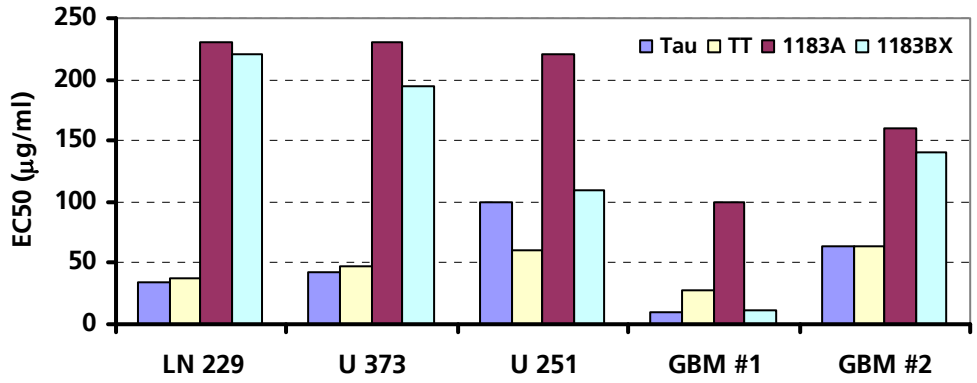

Figure 5. EC_{50} of taurolidine, TT, 1183A, 1183BX in LN229, U373, U251, GBM #1 and GBM #2. Cytotoxicity was measured after 24 hours and data are shown as mean values of three individual experiments.

The overview of Table 1 demonstrates that EC_{50} of taurolidine after an exposure time of 24 hours was always less than 100 µg/ml. The mean EC_{50} was 45 ± 24 µg/ml and the EC_{50} ranged from 30 µg/ml for U373 and U251, the most sensitive cell lines, to 90 µg/ml for U87G, the most resistant cell line. The mean EC_{50} of TT was 101 ± 100 µg/ml, ranging from 37 µg/ml for the most sensitive cell line LN229, to 260 µg/ml for T98G, the most resistant cell line. The mean EC_{50} of 2012, GS208, 1183A and 1183BX always exceeded 100 µg/ml noticeable. At the maximal concentration of 800 µg/ml HG40, E1234 and 2240 were not able to induce a killing rate of 50%, therefore their EC_{50} could not be calculated. For the ex vivo glioblastoma cultures that we have tested, taurolidine and TT were again the most powerful agents. The mean EC_{50} of taurolidine was 36 ± 38 µg/ml and the mean EC_{50} of TT was 45 ± 25 µg/ml. The two ex vivo GBM were also sensitive to 2012 with an EC_{50} of 45 ± 48 µg/ml. 1183A and 1183BX had an EC_{50} greater than 75 µg/ml.

Table 1. Cytotoxicity of taurolidine and its derivates on human glioma cells after 24 and 48 hours of treatment

cell line		Cytotoxicity[a]								
		EC_{50}(μg/ml) 24 h[b]								
	n	Tau	TT	2012	GS208	HG40	E1234	2240	1183A	1183BX
LN229	11	35	37	120	145	>800	>800	>800	230	220
U373	3	30	47	90	100	>800	>800	>800	230	195
U251	3	30	60	98	220	>800	>800	>800	220	110
T98G	3	38	260	155	200	>800	>800	>800	380	360
LN18	3	50	ND	110	195	>800	>800	>800	ND[c]	ND
U87G	2	90	ND	380	390	>800	>800	>800	ND	ND
mean ± SD		45 ± 24	112 ± 100	158 ± 111	208 ± 99	n.a.[d]	n.a.	n.a	265 ± 77	221 ± 104
GBM #1	3	9	27	11	ND	ND	ND	ND	100	12
GBM #2	3	63	63	80	ND	ND	ND	ND	160	140
mean ± SD		36 ± 38	45 ± 25	45 ± 48					130 ± 42	76 ± 90

cell line		Cytotoxicity								
		EC_{50}(μg/ml) 48 h								
	n	Tau	TT	2012	GS208	HG40	E1234	2240	1183A	1183BX
LN229	3	28	ND	78	80	>800	>800	>800	ND	ND
U373	3	9	ND	145	150	>800	>800	>800	ND	ND

[a] Cytotoxicity was assessed after 24 and 48 h of treatment by crystal violet staining
[b] EC50 = effective concentration resulting in 50% cell death compared to untreated control cultures
[c] ND = not done
[d] n.a. = not applicable

4.2 Influence of the caspase inhibitor Z-VAD-FMK and the radical scavenger NAC on taurolidine- and TT- induced cell death

In previous studies it was shown that N-acetyl-L-cysteine (5 mM), a precursor of glutathione, prevents taurolidine- induced cell death. This protective effect is explained by its nucleophilic and antioxidant properties. Therefore it is supposed that taurolidine induces cell killing by induction of reactive oxygen intermediates (ROI) [16, 19].

We confirmed the protective effect of NAC, added at a concentration of 5 mM 20 minutes before treatment. The killing induced by 25 μg/ml and 50 μg/ml taurolidine was reduced by 89% respectively 81% in LN229 glioma cell line (Fig. 6A). Cell killing induced by a high concentration of 100 μg/ml taurolidine could not be prevented. Furthermore, we demonstrated that killing induced by 25 μg/ml TT in the same cell line was likewise

decreased by 68%, but there was no remarkable protective effect of NAC at the killing induced by 50 μg/ml and 100 μg/ml TT (Fig. 6B).

Z-VAD-FMK is a pan-caspase inhibitor that can inhibit induction of apoptosis by binding irreversibly to the effector caspases. It is known that apoptosis induced by FasL is completely inhibited when Z-VAD-FMK was added to the cells one hour before treatment at a concentration of 50 μM. As recently shown in our laboratory caspase activation is not required for taurolidine- induced cell death of glioma cells [19].

Our results verify the presumption that Z-VAD-FMK (10 μM) had no influence neither on taurolidine- nor on TT- induced cytotoxicity. It minimizes the killing induced by 50 μg/ml taurolidine on the LN229 glioma cell line maximal at 10% and there was no reduction of cell death induced by TT (Fig. 6B). To proof the protective effect of Z-VAD-FMK on caspase-dependent cell death, we treated the same cell line, after adding Z-VAD-FMK at a concentration of 10 μM, with Mega FasL at various concentrations (5 to 500 ng/ml). As shown in Figure 6C, Z-VAD-FMK prevented apoptosis induced by Mega FasL completely at all concentrations investigated.

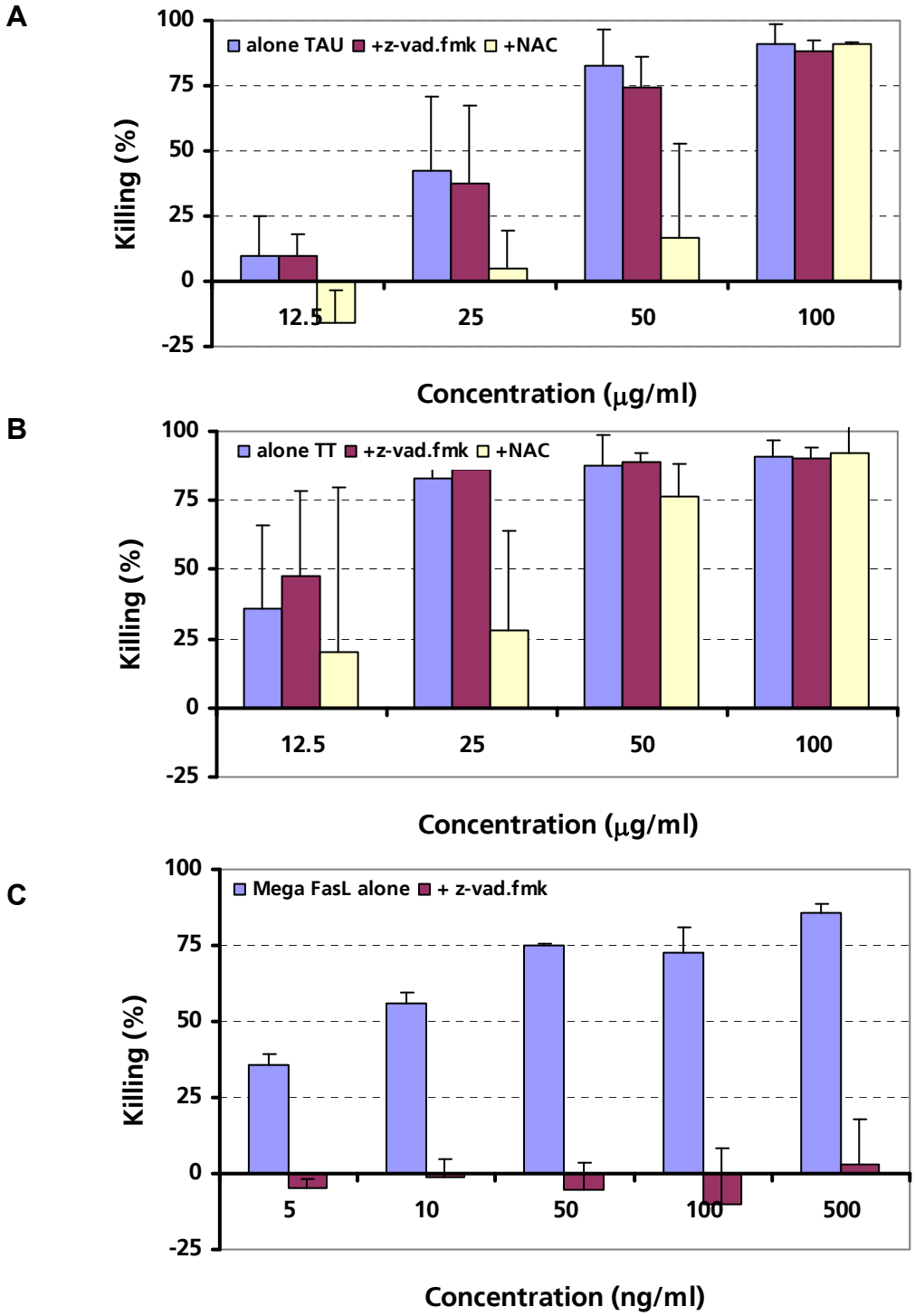

Figure 6. The radical scavenger NAC, but not the pan-caspase inhibitor Z-VAD-FMK prevents taurolidine- or TT- induced killing. The LN229 glioma cells were pretreated with Z-VAD-FMK (10μM) for 60 minutes or NAC (5mM) for 20 minutes and then left untreated or exposed to taurolidine (A), TT (B) and Mega FasL (C) for 24 h. Data are given as mean values ± SD of three independent experiments.

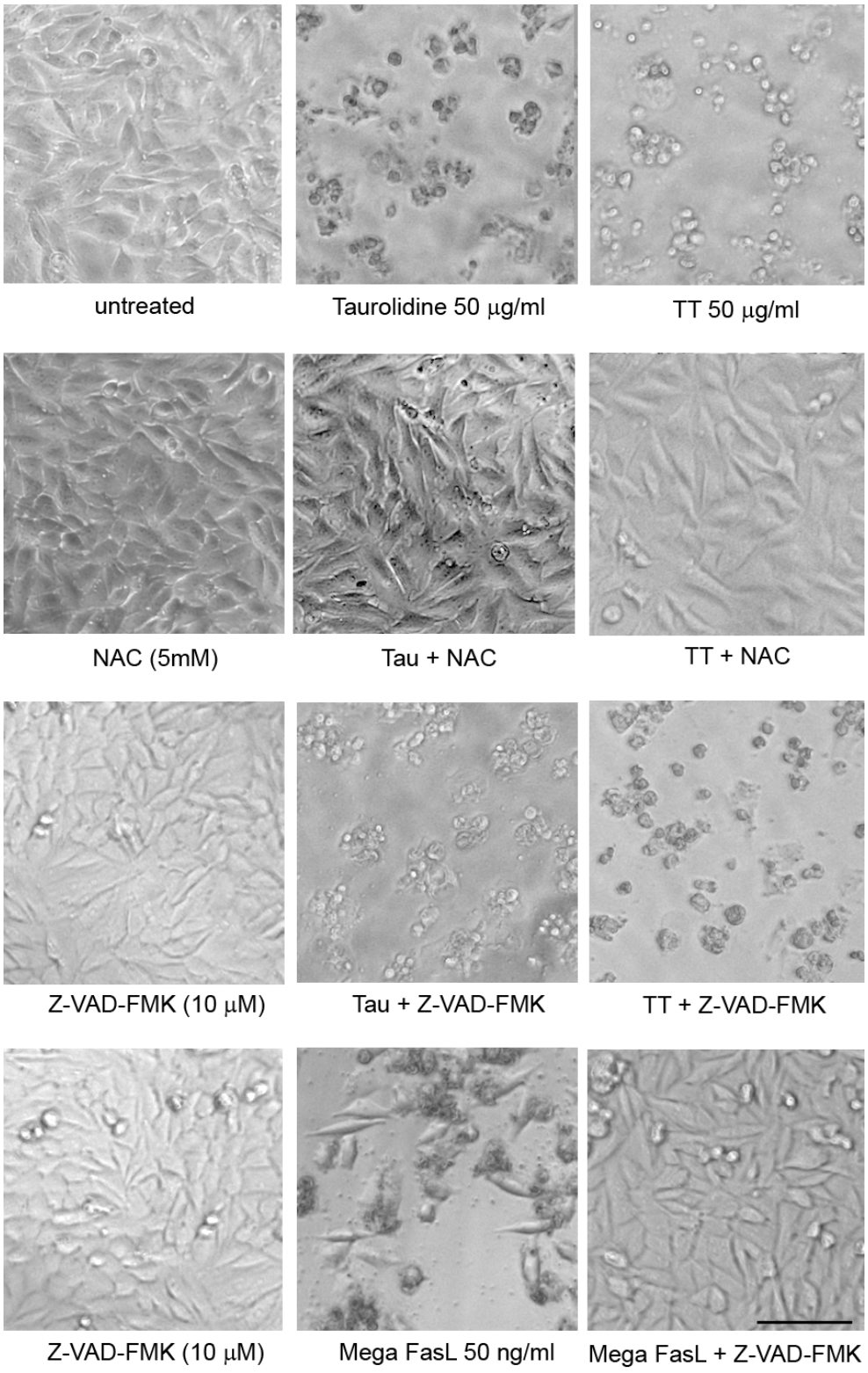

Figure 7. Phase contrast photo-micrographs demonstrating the cytotoxicity induced by taurolidine, TT and Mega FasL on LN229. The cells were left either untreated or pretreated with Z-VAD-FMK (10 μM) or NAC (5mM) before taurolidine or TT exposure (50 μg/ml) for 24h.

4.3 Time- and dose- dependent cytotoxicity of taurolidine and TT on mouse bulk and cancer stem cells

Murine SMA 560 bulk cells were treated with various concentrations of taurolidine and TT (6.25, 12.5, 25, 50, 100, 200 μg/ml) and cytotoxicity was determined after 24 and 48 hours. As shown in Figure 8, cytotoxicity of taurolidine and TT on SMA 560 bulk cells displayed a dose-dependency, but non remarkable time-dependency. The EC_{50} was 19.3 μg/ml for TT and 34.6 μg/ml for taurolidine and did not significantly change between 24 and 48 hours of treatment.

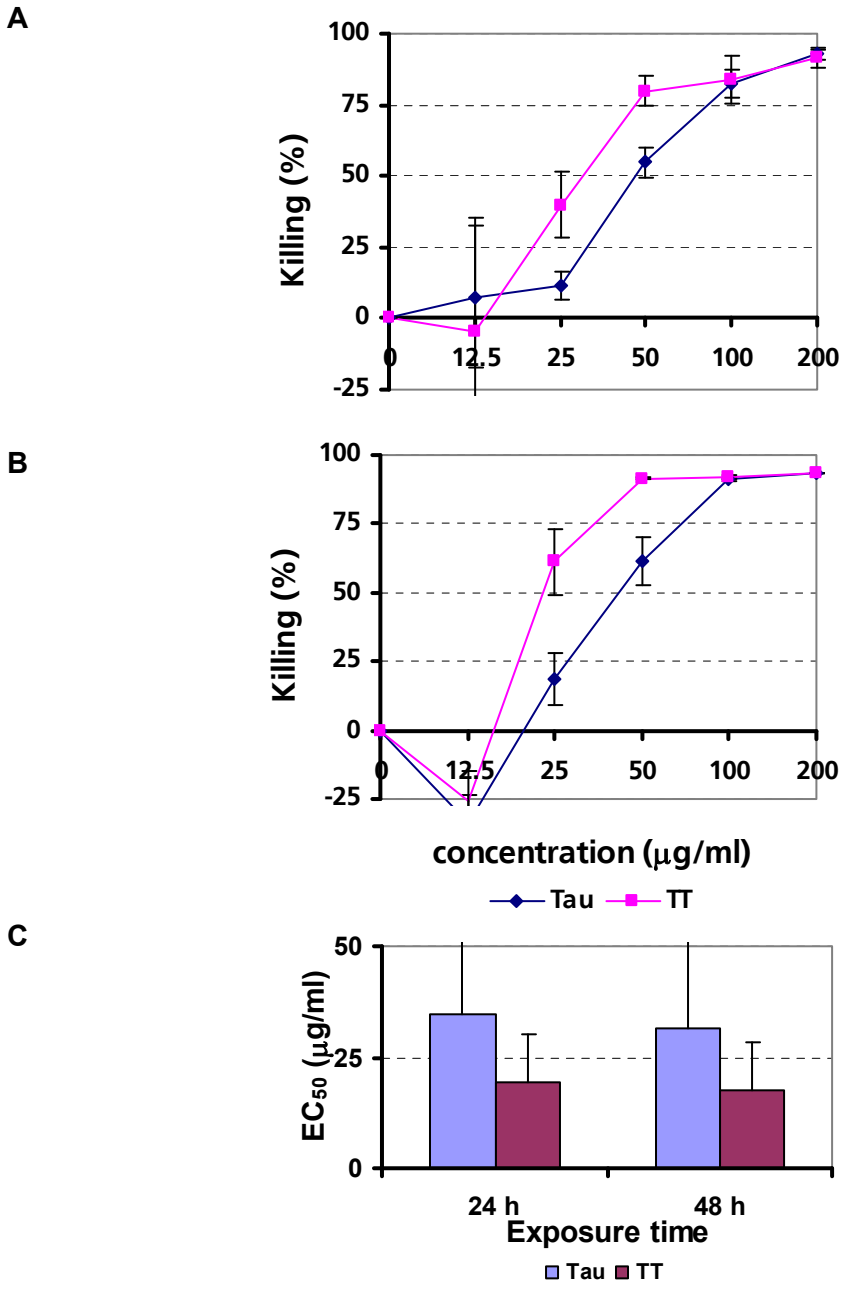

Figure 8. Cytotoxicity in SMA 560 bulk glioma cells after treatment with taurolidine and TT. Cytotoxicity was assessed after 24 h (A) and 48 h (B) of treatment. EC_{50} of taurolidine and TT in the same cell line (C). Data are presented as mean values ± SD of three independent experiments.

After the in vitro generation of CSC from SMA 560, we treated them with taurolidine and TT at the same concentrations (6.25, 12.5, 25, 50, 100, 200 μg/ml). After 7 days of exposure to these agents, cytotoxicity was measured using the AlamarBlue assay. Figure 9 shows that taurolidine and TT generate a dose-dependent cytotoxicity on CSC as well. EC_{50} of TT was 10 μg/ml whereas taurolidine reached EC_{50} at a concentration of 12.5 μg/ml.

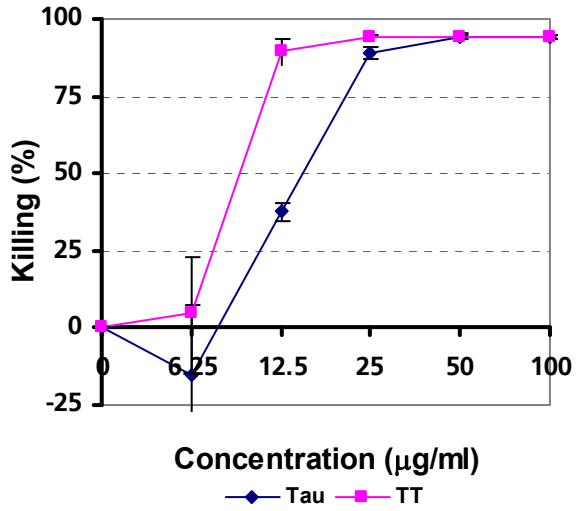

Figure 9. Cytotoxicity of taurolidine and TT on murine SMA 560 glioma CSC. Data are presented as mean values ± SD of two independent experiments.

4.4 Taurolidine and TT induce cell death on human CSC of four different glioblastoma patients

Based on our findings that taurolidine and TT induce cell death of murine CSC, we treated ex vivo human CSC, isolated from glioblastoma tissue as recently described by Galli et al [8]. The same range of concentrations of taurolidine and TT were applied and the cytotoxicity was measured after 7 days of incubation. Figure 10 demonstrates that all glioblastoma CSC (GBM #3, #4, #5 and #6) were sensitive to taurolidine and TT. The mean EC_{50} of taurolidine was 13 ± 2 μg/ml, whereas the mean concentration of TT resulting in 50% cell death was 11 ± 0.9 μg/ml.

We compared the cytotoxic capacity of taurolidine and TT with TMZ on the same four GBM CSC at various concentration of TMZ between 5 μM to 1000 μM. The EC_{50} of TMZ were in the range of 220 to 500 μM, corresponding to a concentration range of 46 to 97 μg/ml. The average EC_{50} was 68.5 ± 26 μg/ml. Interestingly, this concentration was much higher than peak plasma levels of TMZ in patients which was determined to be 13.7 μg/ml (=115 μM) [25].

Table 2. In vitro effect of taurolidine, TT and TMZ on CSC derived from four different glioblastoma patients.

CSC		Cytotoxicity[a]		
		EC_{50} (?g/ml) 24 h[b]		
	n	Tau	TT	TMZ
GBM #3	3	15	10.5	84.4 (435 µM)
GBM #4	2	12.5	12.5	97 (500 µM)
GBM #5	2	14	11	48.5 (250 µM)
GBM #6	3	10	9	44 (230 µM)
mean ± SD		13 ± 2	11 ± 1.4	68.5 ± 26

[a] Cytotoxicity was assessed after 7 days of treatment by AlamarBlue.
[b] EC50 = effective concentration resulting in 50% cell death compared to untreated control cultures.

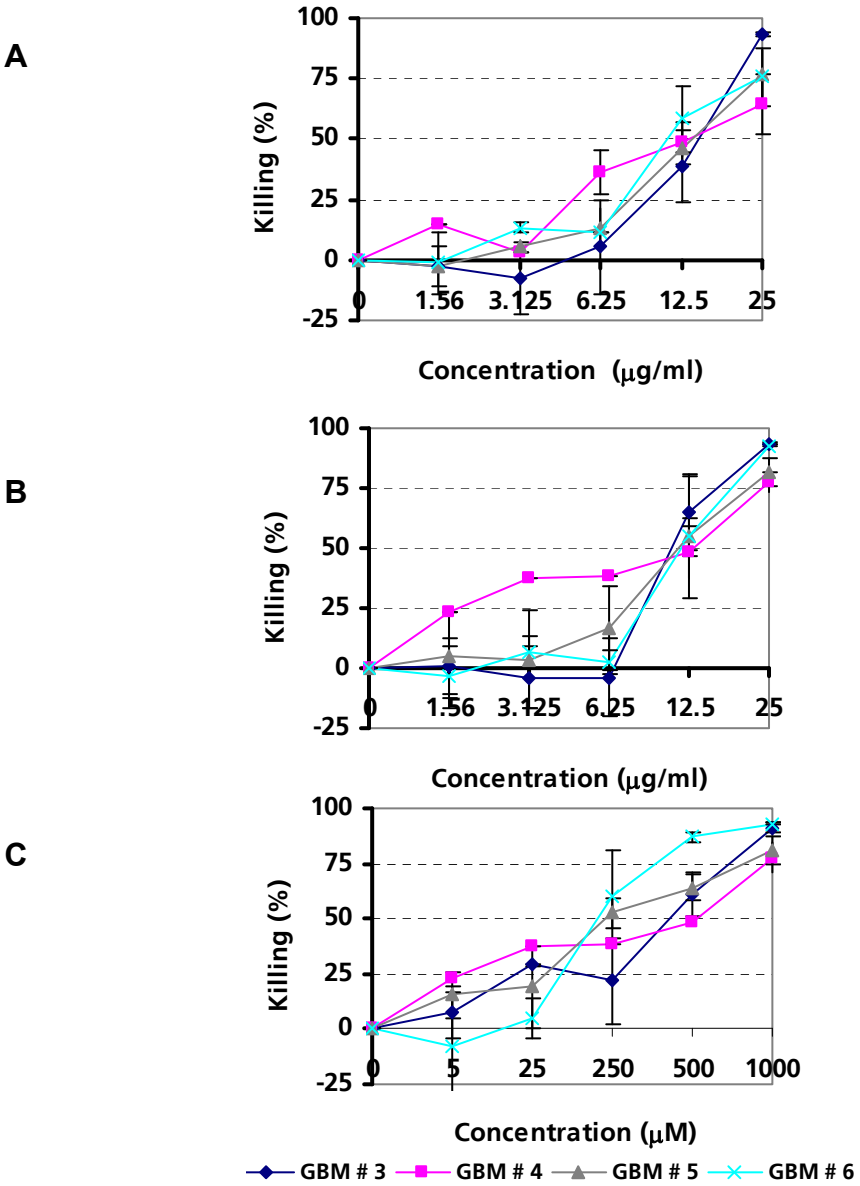

Figure 10. Cytotoxicity induced in CSC of 4 GBM patients (GBM #3, #4, #5 and #6) after treatment with taurolidine (A), TT (B) and TMZ (C). Cytotoxicity was measured after 7 days of treatment with AlamarBlue. Data are presented as mean values ± SD of two to three independent experiments.

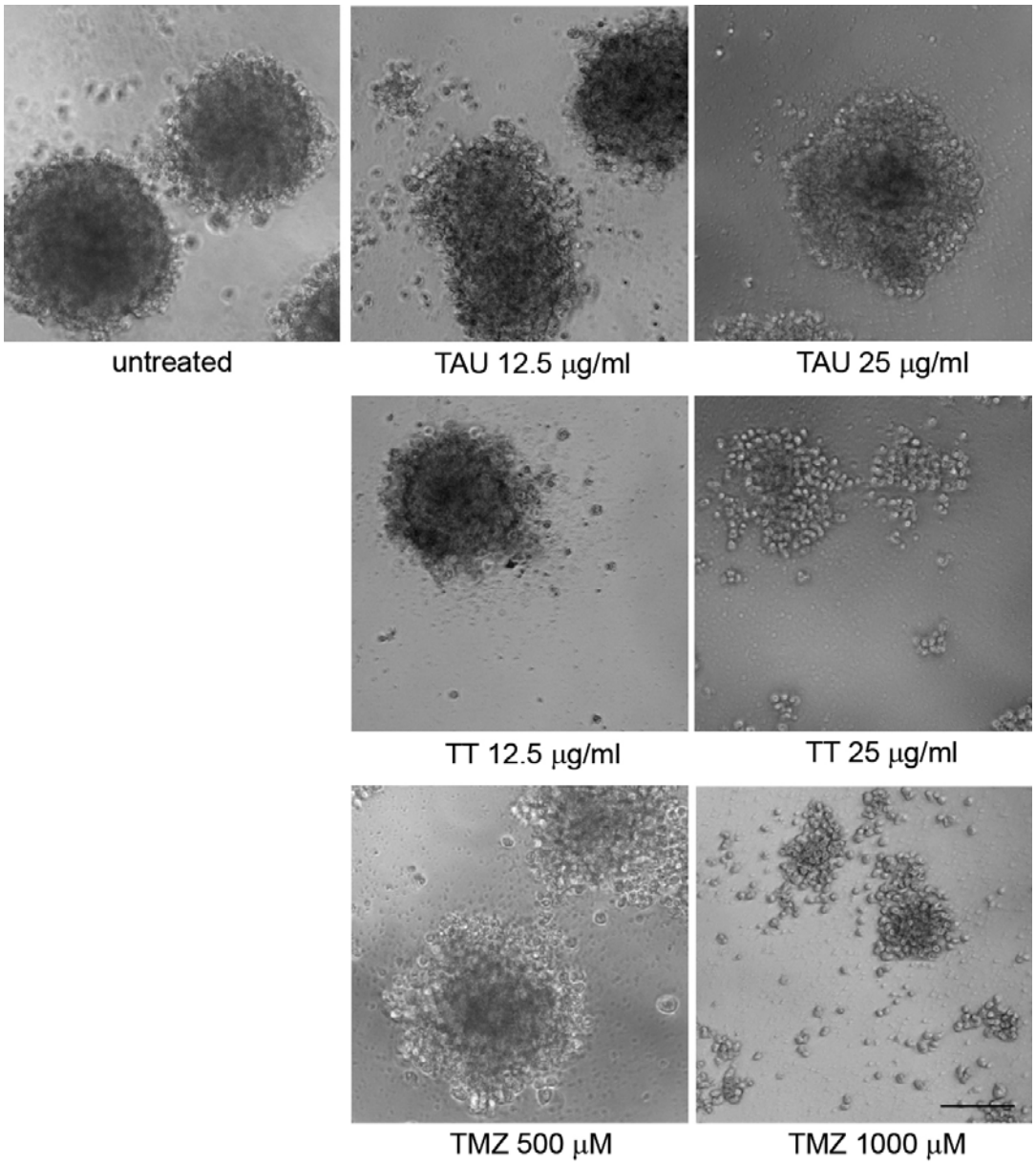

Figure 11. Phase contrast photomicrographs demonstrating the cytotoxicity induced by tauroldine, TT and TMZ on CSC of GMB #6. The cells were left either untreated or treated for 7 days. Bar=100 μm.

4.5 Comparison of the EC_{50} of taurolidine, TT and TMZ on bulk versus CSC of GBM #3

To address the question whether bulk and CSC of the same ex vivo glioblastoma show similiar or different susceptibility to the agents we have tested above, we treated bulk and CSC of GBM #3 with taurolidine, TT and TMZ for four days. As shown in Figure 12, CSC were more sensitive to TT and TMZ. The EC_{50} of CSC is reduced for TT by 51%, for TMZ by 60%, whereas for taurolidine the concentration required to kill 50% of the CSC is increased.

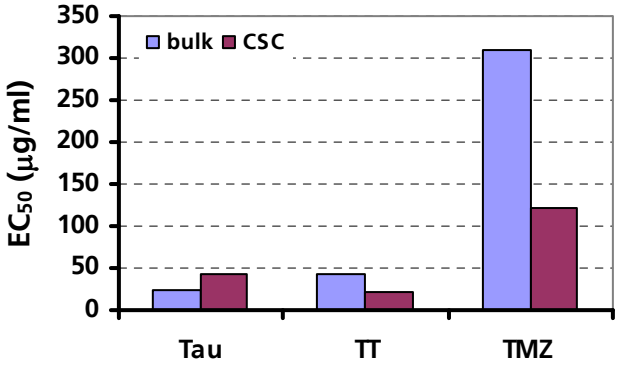

Figure 12. EC_{50} of taurolidine, TT and TMZ in bulk and CSC of GBM #3. Data are shown as mean values of three individual experiments.

4.6 Influence of anti-CXCR1 antibody treatment on human CSC

A recently published study reported that IL-8 receptor blockade selectively targets breast cancer stem cells [24]. In 1997, it was published that IL-8 is up regulated in brain tumour tissue [23]. Therefore, we treated CSC from GBM #3, #4 and #5 with anti-CXCR1 antibody at concentrations of 2.5, 10 and 25 µg/ml. Thereafter, the cells were incubated for 7 days and the cytotoxic effect evaluated. As presented in Figure 11, the antibody treatment induced in GBM #5 about 25% cell death. In the other tested GBM no killing effect has been found.

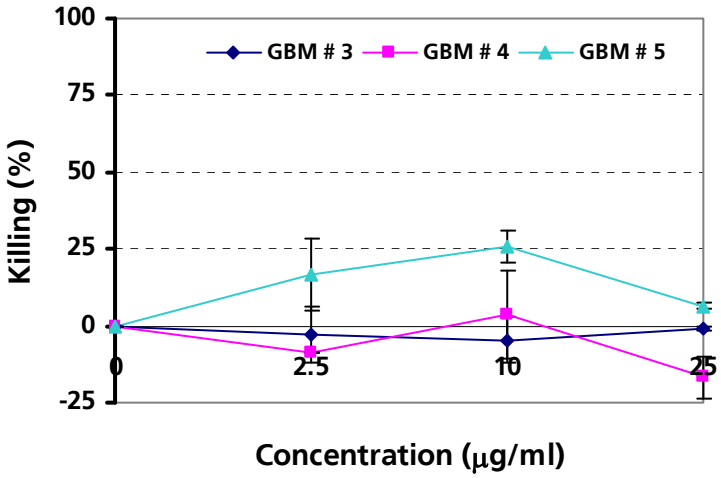

Figure 13. Cytotoxicity in CSC from three different glioblastoma patients (GBM #3 #4 and #5) after treatment with anti-CXCR1 antibody, measured after seven days with AlamarBlue. Data are presented as mean values ± SD of three independent experiments.

4.7 Influence of recombinant IL-8 on the proliferation of human CSC

Since we had no cell death after blocking CXCR1 in GBM #3 and #4, but cell killing induction in GBM #5, we treated GBM #3 and GBM #5 with recombinant IL-8 at concentrations of 2.5, 10 and 50 ng/ml to investigate if external IL-8 would activate the proliferation of human CSC. Figure 14 shows that recombinant IL-8 did neither in GBM #3 nor in GBM #5 stimulate the proliferation. To see whether the negative findings could be explained by the lack of the IL-8 receptor we performed immunofluorescence staining (Fig. 15). As shown in Figure 15, not only CSC (spheres) but also glioblastoma tumour tissue showed a CXCR1 expression of 20-30%.

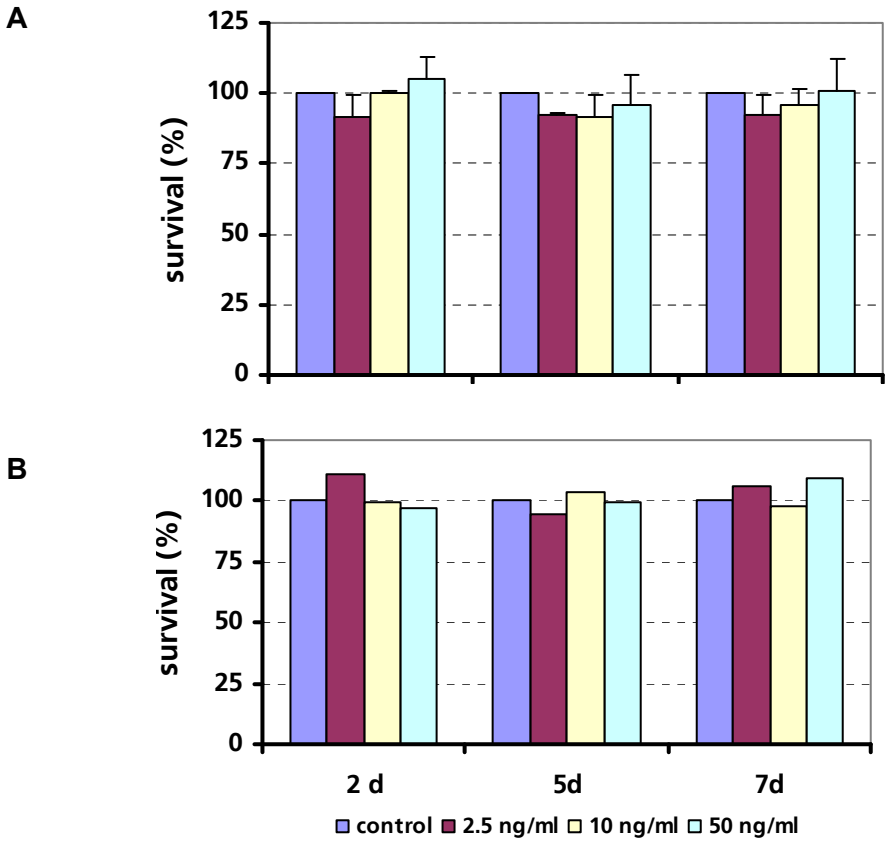

Figure 14. Stimulation of CSC from GBM #3 (A) and GBM #5 (B) after treatment with recombinant IL-8. Proliferation was assessed after 2, 5 and 7 days with the AlamarBlue assay.

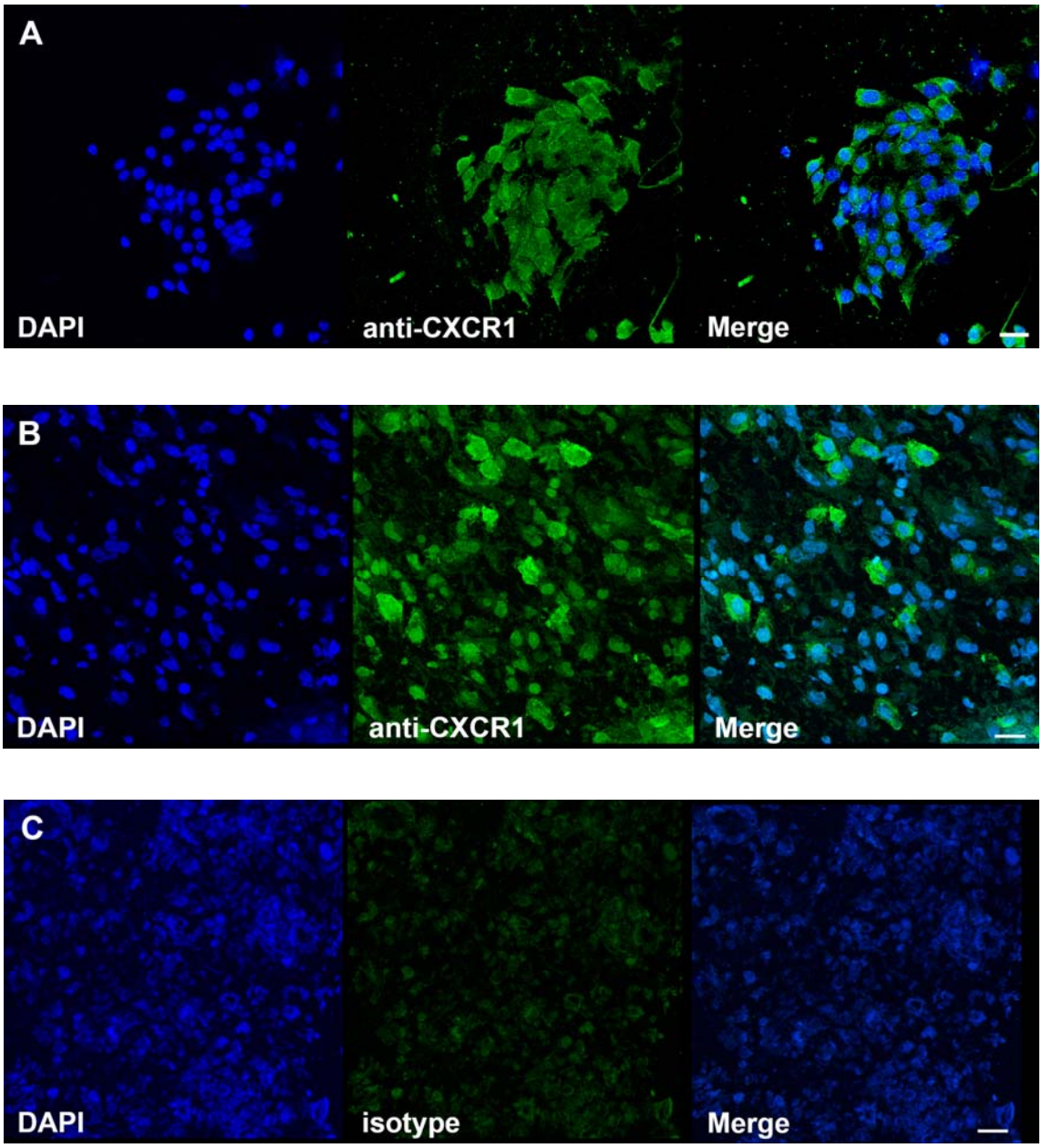

Figure 15. CXCR1 protein expression in spheres and tissue of glioblastoma (GBM #6). Spheres grown on cover slips and stained with anti-CXCR1 (A). Frozen sections of tumour tissue, labeled with anti-CXCR1 (B). Frozen sections of tumour tissue, labeled with an isotype control (IgG2a) (C). DAPI was used to stain cell nuclei. Bars: 20 μm.

5. Discussion

Despite ongoing research, glioblastoma multiforme are still resistant to current surgery, chemo- and radiotherapy. With this study we continued the ongoing research of our laboratory on the antineoplastic activitiy of taurolidine on glioblastoma cells. The goal was to investigate the most powerful derivatives of taurolidine, which were still unknown. We tested taurolidine and eight derivatives on glioma cell lines and ex vivo glioblastoma cells. In addition, we tested the chemosensitivity of cancer stem cells isolated from glioblastomas.

Isolation of the most powerful derivatives

In the first part of this thesis we assessed the antineoplastic potential of taurolidine and all its breakdown products on several glioma cell lines and ex vivo cultured glioblastoma cells. We performed a number of cytotoxicity assays, measuring the susceptibility to these agents. We found that all tested glioblastoma cell lines were sensitive to taurolidine, reaching a mean EC_{50} of 45 μg/ml. With a mean EC_{50} of 101 μg/ml taurultam (TT) posses the highest antineoplastic activity of all intermediates tested. All other derivates were less potent and had therefore a considerably higher EC_{50}. The results for ex vivo glioblastoma cells were similar. Therefore, we assume that TT is mainly responsible for the antineoplastic activity of taurolidine. Bearing in mind that in previous reports on clinical applications of taurolidine the plasma concentrations of taurolidine (or its initial derivatives) ranged from 20 to 100 μg/ml in patients with septicaemia whereas in healthy volunteers taurolidine was examined in a concentration range of 5 to 100 μg/ml [19] and knowing that except for one cell line EC_{50} of taurolidine and TT was lower than 60 μg/ml, we assume that not only taurolidine but also TT are promising therapeutic agents. Since first case reports of intravenous taurolidine treatment in patients with progressive GBM showed a transient improvement in patients' quality of life and partial tumour remission [26], further clinical investigations should be aspired. At the moment encapsulated taurolidine is under clinical investigation with the advantage of a per oral application.

Cell killing mechanism of taurolidine and TT

Our findings are in accordance with earlier experiments performed in our laboratory by Rodak, et al [19] who demonstrated that the pan-caspase inhibitor Z-VAD-FMK, which protects cells completely from FasL-induced apoptosis, did not greatly influence taurolidine-induced cell death of GBM cells. They further proofed that cell death induced by taurolidine can be inhibited by a coincubation with the radical scavenger NAC and concluded that taurolidine induce cell killing by formation of reactive oxygen intermediates (ROI). Further

investigations on this topic showed that oxidative cell damage leads to several cell death pathways such as autophagy, senescene, necroptosis and necrosis [5].
Our results confirm the assumption that TT and all the other derivatives are inducing the same cell death mechanism as taurolidine, namely causing oxidative cell damage as NAC, but not Z-VAD-FMK prevented TT- induced cell death. From the fact that NAC is not able to inhibit cell death induced by TT, concentrations higher than 50 μg/ml, leads to the question whether additional mechanism of cell death are involved. A recently published study that performed a comparative analysis of cell death induction by taurolidine simultaneously in different malignant cell lines proofed that taurolidine provides multifaceted cell death mechanism leading to a cell line specific diversity [27].

Cytotoxicity on CSC and comparision to the effect of TMZ

In the second part of this thesis we examined the chemosensitivity of freshly isolated glioblastoma CSC to taurolidine and TT. Our findings revealed that all CSC were sensitive to taurolidine and TT. The EC_{50} of both agents were in the range of the measured plasma level of taurolidine. In contrast the EC_{50} of TMZ on CSC was higher than its peak plasma level. The 2-fold higher EC_{50} of GBM #3 and #4 is probably due to their non methylated MGMT status.
Based on these findings we wanted to compare the susceptibility of CSC versus bulk tumour cells. Our results have shown that CSC are more sensitive to taurolidine and TT than bulk cells. Interestingly, TMZ was also more potent on CSC which is in accordance with a study published by Beier, et al [16]. The reason for the preferential depletion of CSC is not elucidated until now. These findings will be evaluated in future experiments in an orthotopic glioma mouse model using SMA 560 CSC and bulk cells.

Impact of CXCR1 targeting by antibodies or IL-8 on CSC

Recently it has been reported that blockage of CXCR1 by an antibody or by the pharmacological inhibitor repertaxin induced CSC death in breast cancer tissue. From the fact that glioblastoma cells are expressing IL-8 and their corresponding receptors, we examined the influence of anti-CXCR1 antibody on CSC. One out of three tested CSC showed a significant cell death whereas the others were resistant. In another set of experiments we tested whether IL-8 added to the CSC cultures could increase the sphere initiation capacity. From the fact that this was not the case, we analyzed CSC on their CXCR1 expression by confocal microscopy laser scanning and found a substantial number of positive cells. This suggests that the signalling pathway in CSC of GBM might be different than in CSC of breast cancer.

Taken together, our study showed that taurultam is the most powerful derivative of taurolidine among the eight tested ones and induces cell death in glioblastoma bulk and CSC by causing oxidative damage leading to cell death. Since all four CSC of the glioblastomas tested are more sensitive than the bulk cells to taurolidine and TT and chemoresistance of CSC is responsible for the recurrence of GBM, taurultam should be investigated in a clinical trial.

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8. Curriculum vitae

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